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MODULATION OF IgE-BINDING PROPERTIES OF TREE POLLEN ALLERGENS BY SITE-DIRECTED MUTAGENESIS

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1.INTRODUCTION

In the last few years, the use of recombinant DNA techniques for the characterization of atopic allergens offered new aspects for diagnosis and therapy of Type I allergies (1). It has made it possible to produce large quantities of well characterized "wild-type" recombinant allergens, or first-generation recombinant products, and many of them are now being developed for diagnosis and possibly therapy of Type I allergies. For instance, recombinant Asp f 1 expressed in *E. coli* was successfully used for serologic and clinical diagnosis of A. fumigatus allergy (2). It has been demonstrated that the use of two recombinant birch pollen allergens, Bet v 1 and Bet v 2 (profilin), allows accurate *in vitro* (ELISA, immunoblots) and *in vivo* (skin prick test, intradermal test) diagnosis of birch pollen allergy (3,4). In addition, recombinant Bet v 1 could be efficiently used for identifying food cross-sensitization induced by Bet v 1-related proteins (4). These studies demonstrate that recombinant allergens are adequate tools for in vivo and in vitro allergen-specific diagnosis, which might be considered as an important step towards allergen-specific therapy. Presently, specific-immunotherapy is performed using natural allergen extracts that may contain, besides the desired allergen, other unwanted components.

In addition to their use for characterization of allergens, recombinant DNA techniques also offered the unique possibility of arbitrarily altering the nucleotide sequence of a gene and, subsequently, the sequence of the encoded protein in order to produce novel "mutant" proteins, or second generation recombinant products, displaying altered proper-

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ties. Previously we have shown that isoforms of Cor a 1, the major hazel pollen allergen, displayed striking differences in their ability to bind IgE from allergic patients (5). Since these isoforms showed high amino acid sequence similarity, we speculated that the differences in IgE-binding was a result of sequence dissimilarities. In particular, the exchange at position 10 from a threonine in Bet v 1a and Cor a 1/11 isoform to proline in Cor a 1/16 isoform seemed to correlate to its lower IgE-binding capacity. In this study, we have tested this hypothesis using a PCR-based site-directed mutagenesis approach to produce a single amino acid exchange in the Cor a 1/16 isoallergen.

2. MATERIALS AND METHODS

2.1. DNA Constructs

The cDNAs coding for Bet v 1a (6) and Cor a 1/16 (5) were cloned in the pMW175 expression vector (7). The amino acid exchange at position 10 (Pro→Thr) in Cor a 1/16 was engineered by PCR-mediated mutagenesis using the following primers:

NcoI mutagenic primer 5'-GGGCCATGGGTGTTTTCAATTACGAGGTT-GAGACCACCTCCGTT-3', base exchanged indicated in bold; NcoI site is underlined.

EcoRI primer 5'-CCCGAATTCTTAGTTGTATTCAGCAGAGTGTGCGAA-3', EcoRI site is underlined.

The PCR was performed with 1 ng template (pMW175/Cor a 1/16 construct) and 1 μM of each primer, using 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 42°C, and 1 min extension at 72°C. The PCR product was digested with NcoI and EcoRI and subcloned in the pMW175 expression vector. The resulting plasmids were used to transform competent *E. coli* BL21 cells. All PCR amplified products were sequenced according to the dideoxi chain termination method (8).

2.2. Expression of Cor a 1/16, Cor a 1/16T10, and Bet v 1a

E. coli BL21 cells containing the pMW175/Bet v 1a, pMW175/Cor a 1/16, and pMW175/Cor a 1/16T10 plasmids were grown and expression of the recombinant allergens induced by adding IPTG to a final concentration of 1 mM. After incubation at 37°C for 6 h, cells were harvested by centrifugation and lysed by repeated freeze-thaw cycles.

2.3. SDS-Page and Immunoblots

E. coli lysates of recombinant Cor a 1/16, Cor a 1/16T10, and Bet v 1a or purified proteins were analyzed by SDS-PAGE according to the method of Laemmli (9), using 15% acrylamide gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. For immunoblot analysis, proteins were separated by 15% SDS-PAGE and electroblotted onto nitrocellulose membranes. Immunoblots using a monoclonal anti-Bet v 1, BIP 1, were performed as described previously (10). IgE immunoblots were carried out using sera from birch pollen allergic patients. Bound IgE was detected using ^{125}I-rabbit anti-human IgE. *E. coli* lysates harboring the plasmid without insert were used as a control. In all experiments, reagents, and cell lysates were from identical batches and were used in the same concentrations. Autoradiography was performed at -70°C for 12–48 h with intensifying screens.

2.4. Purification of Recombinant Allergens

rBet v 1a, rCor a 1/16, and rCor a 1/16T10 proteins were purified from crude bacterial lysates by chromatofocusing and reversed-phase HPLC (10). Purified proteins were analysed by SDS-PAGE according to the method of Laemmli (9) and visualized by staining with Coomassie Brilliant Blue R-250.

2.5. Trypsin Treatment, and Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry (MALDI-MS) of Cor a 1/16 and Cor a 1/16T10 Proteolytic Fragments

Purified Cor a 1 proteins (100 µg in distilled water) were heated for 20 min at 95°C and diluted with an equal volume of 0.2 M NH_4HCO_3. One microgram of trypsin was added and the mixture incubated at 37°C for 2 h. Afterwards, trypsin was added again, and incubation continued for an additional 4-h period. The reaction was stopped by adding 1/10 vol of trifluoracetic acid and dried *in vacuo*. The resulting peptide mixtures were subjected to MALDI-MS analysis using the HP G2025A system equipped with a nitrogen laser.

2.6. T-Cell Proliferation Assays

Isolation of Bet v 1-specific T-cell clones from the peripheral blood of birch pollen allergic patients (as indicated by typical case history, positive skin tests, and positive RAST) and proliferation assays were done as previously described (11).

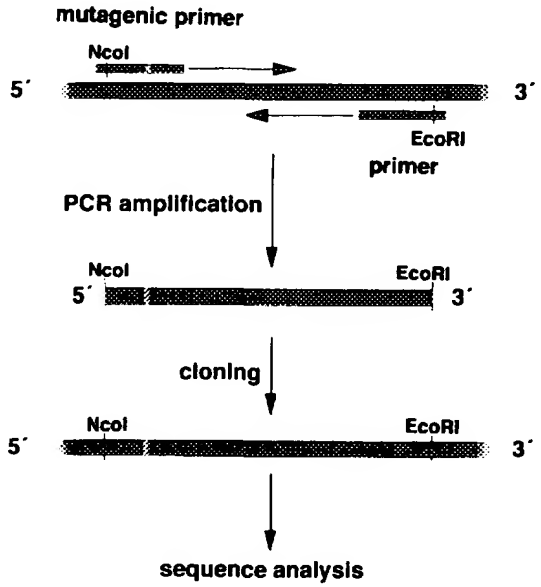

Figure 1. PCR mutagenesis of the Cor a 1/16 cDNA. The 5' Nco mutagenic primer and the 3' EcoRI primer were used in a PCR with the Cor a 1/16 cDNA.

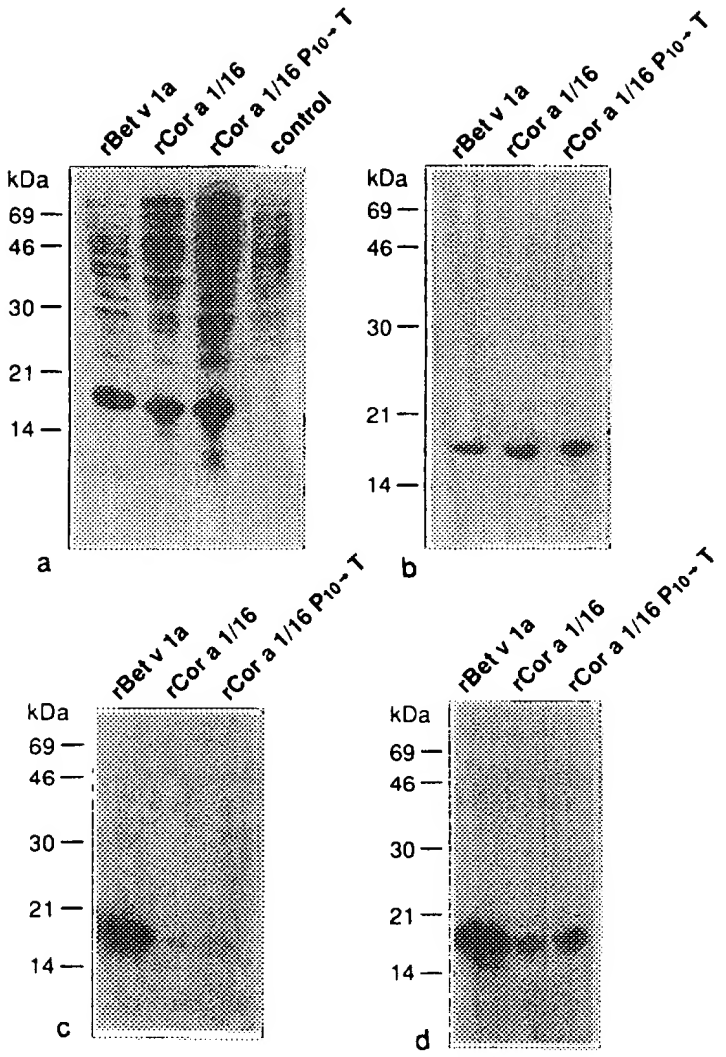

Figure 2. Expression, purification and immunoblot analysis of Cor a 1/16T10 mutant protein. (a), Coomassie-stained 15% SDS-polyacrylamide gel of lysates of *E. coli* BL21 host strain containing Cor a 1/16, Cor a 1/16T10, and Bet v 1a pMW175 expression plasmids. Control, host strain BL21 lysate containing the pMW175 expression vector without an insert. (b), Coomassie-stained 15% SDS-polyacrylamide gel of purified rCor a 1/16, rCor a 1/16T10 and rBet v 1a. (c) and (d), Immunoblots of purified rCor a 1/16, rCor a 1/16T10, and rBet v 1a probed with BIP 1, a monoclonal anti-Bet v 1, and with a polyclonal anti-rBet v 1a, respectively.

3. RESULTS AND DISCUSSION

3.1. Expression, Purification, and Mass Spectrometry Analysis of Cor a 1/16 Proteins

In this study, we have used PCR-mediated mutagenesis to generate a mutant of the Cor a 1/16 allergen. A mutant primer was used that had the proline codon at amino acid position 10 replaced by a threonine codon. The strategy for the construction of this Cor a 1/16 mutant (Cor a 1/16T10) is outlined in Figure 1.

The cDNAs coding for Bet v 1a, Cor a 1/16 and Cor a 1/16T10 were subcloned in the pMW175 expression vector and high-levels of recombinant non-fusion proteins were produced by induction with IPTG. Figure 2A shows a Coomassie-stained gel of expressed Bet v 1a, Cor a 1/16, and Cor a 1/16T10 proteins.

The recombinant proteins were purified from crude bacterial lysates using chromatofocusing and reversed-phase HPLC. The proteins appeared homogeneous as determined by SDS-PAGE and Coomassie-staining (Figure 2B).

In order to confirm at the protein level the sequence of the Cor a 1/16T10 mutant allergen, purified rCor a 1/16 and rCor a 1/16T10 were digested with trypsin and the proteolytic fragments subjected to MALDI-MS. Nine peptides were detected for both Cor a 1/16 and Cor a 1/16T10, and their molecular weights were determined from the obtained spectra (Table 1). The observed mass signals could be easily matched with the molecular weight of peptides predicted from the amino acid sequence deduced from the published Cor a 1/16 sequence (T1, T4-T6, T9–10, T12, T18–19). These peptides covered about 70% of the Cor a 1/16 sequence. All peptides detected by MALDI-MS of rCor a 1/16 digests were also detected in rCor a

Table 1. Mass determination of tryptic fragments T1-T19 of rCor a 1/16T10. Theoretical m/z values give the calculated masses of the peptides plus one proton $[M+H]^+$

Fragment	*Sequence*	*m/z*	
		theor.	*observed*
T1	GVFNYEVETPSVISAAR	1840.03	1839.6
T1 P_{10}→T	GVFNYEVETTSVISAAR	1844.02	1843.2
T2	LFK	407.53	-
T3	SYVLDGDK	896.96	-
T4	LIPK	470.62	470.3
T5	VAPQAITSVENVGGNGGPGTIK	2067.29	2067.3
T6	NITFGEGSR	981.04	981.2
T7	YK	310.36	-
T8	YVK	409.49	-
T9	ERVDEVDNTNFK	1466.54	1466.6
T10	YSYTVIEGDVLGDKLEK	1929.96	1929.4
T11	VCSELK	678.81	-
T12	IVAAPGGGSTLK	1071.25	1071.2
T13	ISSK	434.50	-
T14	FHAK	502.58	-
T15	GDHEINAEEMK	1273.35	-
T16	GAK	275.32	-
T17	EMAEK	607.69	-
T18	LLR	401.52	401.5
T19	AVETYLLAHSAEYN	1581.71	1581.7

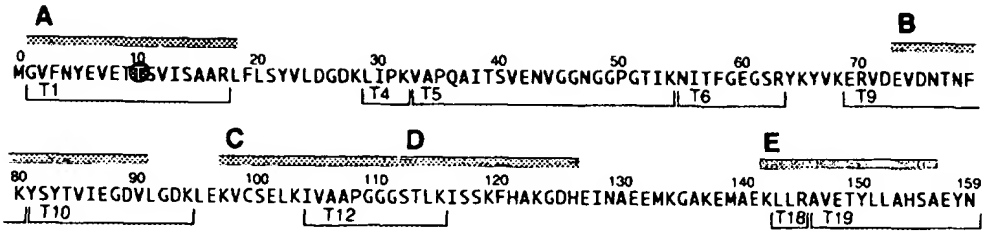

Figure 3. Tryptic peptides of rCor a 1/16T10 (T1-T19) identified by MALDI-MS. The positions of peptides stimulating specific T cell clones (A-E) are indicated by boxes above the sequence.

1/16T10 digests, except for the signal at m/z 1839 corresponding to the N-terminal peptide T1 (Table 1). According to the Cor a 1/16T10 cDNA sequence, peptide T1 should contain the single amino acid exchange (Pro → Thr) when compared to Cor a 1/16. In tryptic digests of rCor a 1/16T10 a signal at m/z 1843 (T-P10 → T) corresponded exactly to the expected mass of T1 with an exchange of proline for a threonine (Table 1). Figure 3 shows the recorded mass signals of rCor a 1/16T10 proteolytic digests mapped onto the cDNA-derived Cor a 1/16T10 sequence according to their molecular mass and enzyme specificity. Thus, the single amino acid substitution engineered in the Cor a 1/16 cDNA to produce Cor a 1/16T10 was also confirmed at the protein level by mass spectrometry analysis of proteolytic digests of rCor a 1/16T10 mutant protein.

3.2 Immunological Properties of Cor a 1/16T10

To evaluate the antibody-binding properties of purified rCor a 1/16T10 mutant protein in comparison to rCor a 1/16 and rBet v 1a, we performed immunoblotting experiments.

Figures 2C and 2D show immunoblots of the purified proteins using a monoclonal anti-Bet v 1 antibody, BIP 1, (Fig. 2C) and a rabbit anti-rBet v 1a serum (Fig. 2D). Both antibodies showed strong reactivity to rBet v 1a. In contrast, BIP 1 did not react with rCor a 1/16 or with rCor a 1/16T10, and the polyclonal anti-Bet v 1a showed a weak reactivity to both rCor a 1/16 and rCor a 1/16T10 proteins.

Immunoblots experiments using sera from birch pollen allergic patients showed remarkable differences in the IgE-binding properties of rCor a 1/16 and rCor a 1/16T10. Figure 4 shows the IgE-binding patterns of rBet v 1a, rCor a 1/16, and rCor a 1/16T10 using sera from five birch pollen allergic patients. All patients showed a marked increase in IgE-binding to rCor a 1/16T10 mutant protein compared to wild type rCor a 1/16, except patient 2. For patients 1, 4, and 5, the replacement of proline at position 10 by a threonine resulted in a change of the antibody-binding pattern from "no-binding" to strong IgE-binding. Interestingly, rCor a 1/16T10 mutant protein in some cases displayed higher IgE-binding activity than rBet v 1a (patients 1, 2, 3, and 5).

Presently, there are no data available on the 3D-structure of Bet v 1 or homologous proteins. Also, there are no precise informations available about IgE-binding motif(s) on the Bet v 1 or Bet v 1-related allergens. However, there are indications that IgE-binding structures on the Bet v 1 molecule might be determined by the protein conformation (10).

Among all standard amino acids, proline seems to occupy a unique position. Proline imposes strong conformational constraints on the peptide chain because the side-chain is cyclized back onto the backbone amide position. When present inside an alpha-helix, the possibility of making hydrogen bonds to the preceding turn is hindered and a kink of 20°

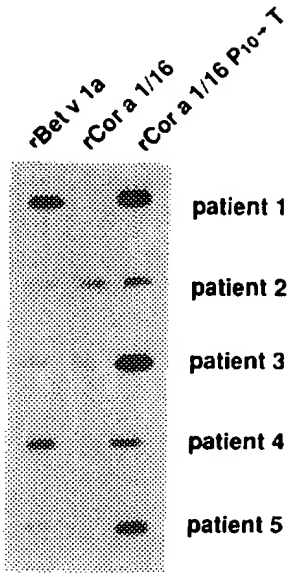

Figure 4. IgE-binding to rCor a 1/16T10, rCor a 1/16, and rBet v 1a. Immunoblot experiments showing serum IgE-reactivity of 5 birch pollen allergic patients.

or more will be introduced in the alpha-helix. In addition, proline can introduce structural heterogeneity since the X-proline (X being any amino acid) bond can assume either the stereoisomeric cis or trans conformation (for a review see 12).

Taking into consideration the exceptional properties of proline, it is possible that the substitution in Cor a 1/16 of proline-10 for a threonine residue might cause conformational changes with the result of a dramatic increase in its IgE-binding activity. This correlates well with the fact that Bet v 1a, which displays high IgE-binding activity, has a threonine at position 10. It will be interesting to test whether the substitution of threonine-10 in Bet v 1a for a proline will lower its IgE-binding activity.

3.3 Activation of Allergen-Specific T Cell Clones

The ability of rCor a 1/16T10 to activate allergen-specific T cell clones was evaluated using Bet v 1a-specific T cell clones. We tested fourteen clones that were established from the peripheral blood of birch pollen allergic patients and were shown to recognize distinct epitopes (A-E) scattered over the whole Bet v 1a molecule (Fig. 5) (ref. 11). Seven of these Bet v 1a-specific T cell clones also recognize the corresponding sequence on Cor a 1/16. The epitope recognized by the clone RR9 comprises the amino acid substitution on rCor a 1/16T10. This clone reacted with both rCor a 1/16 and rCor a 1/16T10 mutant protein. Except for the clone WD25, all other clones reacting with rCor a 1/16 also reacted with rCor a 1/16T10. It is not clear why this clone failed to proliferate in response to rCor a 1/16T10. As shown in Fig. 5, this clone recognizes an epitope (epitope E) corresponding to the C-terminal region (142–156) of Bet v 1a and Cor a 1/16. The possibility of a mutation in the peptide epitope recognized by this clone can be ruled out since MALDI-MS analysis of proteolytic fragments of rCor a 1/16T10 confirmed the structural integrity of the region corresponding to epitope E (see Fig. 3 and Table 1). It is conceiv-

TCC	EPITOPE	rBet v 1a	rCor a 1/16	rCor a 1/16 P_{10}→T
BE6	A	+	-	-
WD27		+	-	-
RR9		+	+	+
MH23	B	+	-	-
WF22		+	++	++
WF20		+	-	-
MS2	C	+	-	-
WF17		+	-	-
TF1		+	-	-
WF38	D	+	+	+
RR4	E	+	+	+
WD24		+	+	+
TF10		+	+	+
WD25		+	+	-

Figure 5. Proliferative responses of human Bet v 1-specific T cell clones (TCC) to rCor a 1/16, rCor a 1/16T10, and rBet v 1a. On top, the black bar represents the Bet v 1a amino acid sequence. The positions of the epitopes (A-E) recognized by the TCC are indicated by boxes above the Bet v 1a sequence.

able that the single amino acid exchange at position 10 in Cor a 1/16T10 can affect the conformation of the protein to an extent that the processing by antigen presenting cells is different and a non-reactive peptide is created or that the epitope is destroyed. In this respect, Finnegan and Amburgey (13) showed that a single amino acid change in the staphylococcal nuclease protein affects the structure of the processed peptides in such a manner that stimulatory determinants are no longer presented to certain T cell clones.

4. CONCLUSIONS

The results presented here suggest that it is possible to modulate the IgE-binding properties of tree pollen allergens by single amino acid substitutions at crucial positions. This finding makes it possible to develop second-generation of recombinant allergens with antibody-binding properties specifically modulated for diagnosis and for therapy.

Following this line, we are presently testing the effect of single amino acid exchanges on the IgE-binding properties of Bet v 1a, which were based on the patterns of amino acid substitutions of Bet v 1 isoforms displaying low IgE-binding properties.

The identification of positions crucial for IgE binding might be facilitated by cloning and sequencing isoforms of a particular allergen and determining their IgE-binding properties. In addition, isoforms with low or no IgE-binding activity could be useful tools for defining IgE-binding structures on allergens.

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